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Pathological Tau Promotes Neuronal Damage by Impairing Ribosomal Function and Decreasing Protein Synthesis

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Pathological Tau Promotes Neuronal Damage by Impairing Ribosomal Function and Decreasing Protein Synthesis

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One of the most common symptoms of Alzheimer's disease (AD) and related tauopathies is memory loss. The exact mechanisms leading to memory loss in tauopathies are not yet known; however, decreased translation due to ribosomal dysfunction has been implicated as a part of this process. Here we use a proteomics approach that incorporates subcellular fractionation and coimmunoprecipitation of tau from human AD and non-demented control brains to identify novel interactions between tau and the endoplasmic reticulum (ER). We show that ribosomes associate more closely with tau in AD than with tau in control brains, and that this abnormal association leads to a decrease in RNA translation. The aberrant tau–ribosome association also impaired synthesis of the synaptic protein PSD-95, suggesting that this phenomenon contributes to synaptic dysfunction. These findings provide novel information about tau-protein interactions in human brains, and they describe, for the first time, a dysfunctional consequence of tau–ribosome associations that directly alters protein synthesis.

Key words: Alzheimer; ribosome; RNA; tau; tauopathies; translation

Significance Statement

Despite the identification of abnormal tau–ribosomal interactions in tauopathies >25 years ago, the consequences of this association remained elusive until now. Here, we show that pathological tau associates closely with ribosomes in AD brains, and that this interaction impairs protein synthesis. The overall result is a stark reduction of nascent proteins, including those that participate in synaptic plasticity, which is crucial for learning and memory. These data mechanistically link a common pathologic sign, such as the appearance of pathological tau inside brain cells, with cognitive impairments evident in virtually all tauopathies.

Introduction

Aberrant accumulation of tau is associated with the etiology of >18 known neurodegenerative diseases collectively termed tauopathies. Alzheimer's disease (AD), the most common tauopathy, affects >36 million people world-wide (Alzheimer's Association, 2014). A common and early symptom in tauopathic patients is progressive memory loss. There is no cure for tauopathies, and current therapeutics stave off symptoms only temporarily. This is partly due to limited understanding of the molecular mechanisms linking tau and disease onset.

Synaptic function depends on constant protein synthesis; therefore, neurons are especially vulnerable to chronic attenuation of RNA translation (Moreno et al., 2012). Although, transient suppression of translation is an adaptive cellular response to endoplasmic reticulum (ER) stress (Harding et al., 1999), chronic inhibition of RNA translation contributes to

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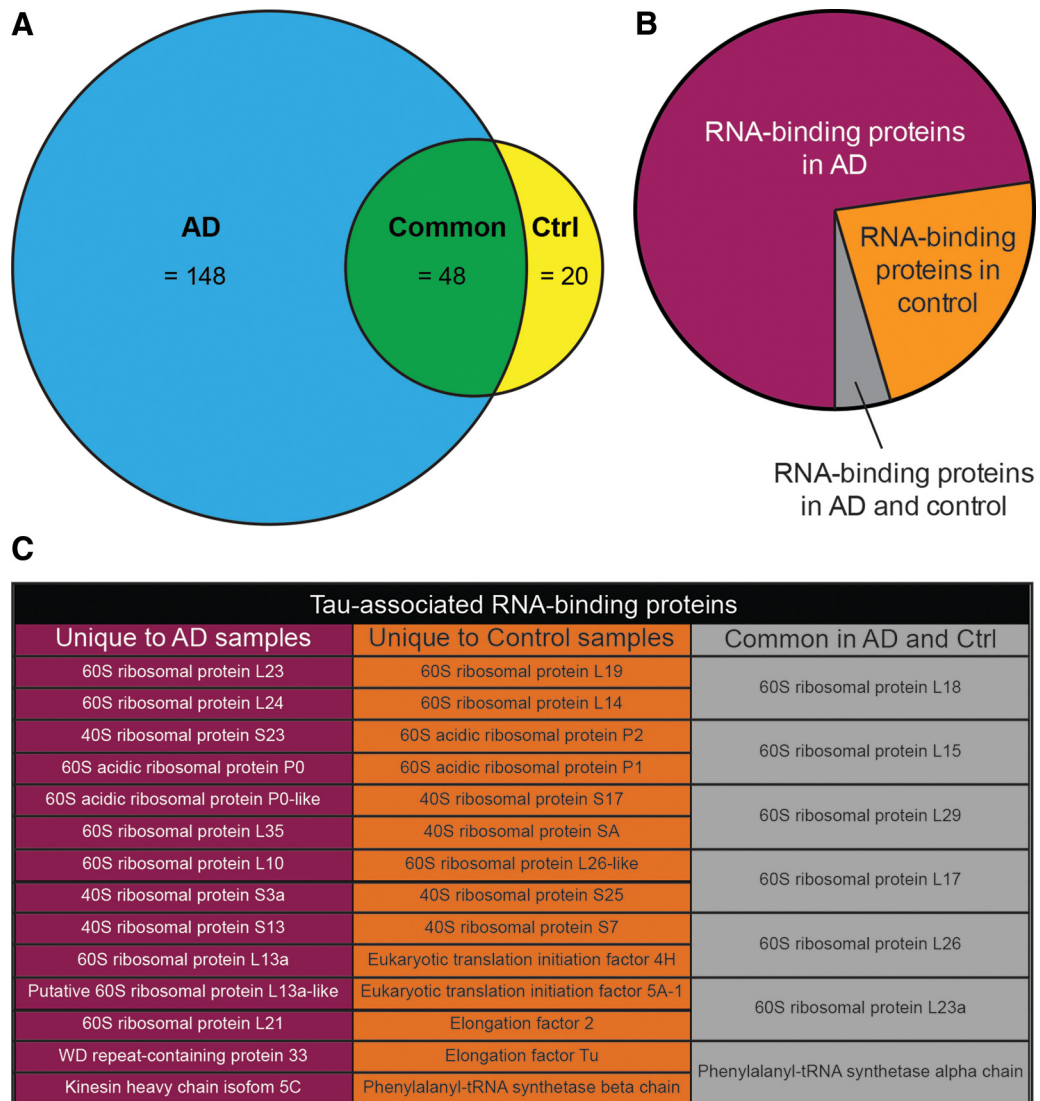


Figure 1. Tau associates with ER proteins differentially in AD versus control brains. **A**, Venn diagram showing the number of tau-associated ER proteins unique to AD brains (blue), unique to control brains (yellow), and common to both (green). A major group of proteins that was identified corresponded to RNA-binding proteins. **B**, Pie chart showing relative abundance of tau-associated, RNA-binding proteins identified in human AD and control brains by LC-MS/MS. **C**, List of proteins identified in **B**.

the pathogenesis of multiple neurodegenerative disorders, including tauopathies (Vanderweyde et al., 2012; Abisambra et al., 2013b; Ash et al., 2014). Pronounced ribosomal deficiencies appear in regions where tau pathology is evident (Ding et al., 2005), yet the link between tau and ribosomal function has not been established.

Protein synthesis drives memory formation (Duvarci et al., 2008). The fact that progressive memory loss is common to virtually all tauopathies suggests that ribosomal dysfunction could be an underlying mechanism leading to clinical symptoms. Indeed, tau binds to ribosomes in the brain, and this interaction is enhanced in tauopathic brains (Papasozomenos and Binder, 1987; Piao et al., 2002).

Here we show that ribosomes associate with pathological and nonpathological tau. Consequently, ribosomal function becomes impaired and global protein synthesis is reduced including the synaptic protein PSD-95. These data suggest that tau-mediated ribosomal dysfunction is a common pathogenic process that affects synapses leading to cognitive impairment.

Materials and Methods

Human brain samples. Human samples were obtained from the University of Kentucky (UK) Alzheimer's Disease Center. Sample collection and experimental procedures involving human tissue were in compliance with the UK Institutional Review board. Samples from Brodmann areas 21/22 were used. AD tissues were scored as Braak V (female, 93-year-old), VI (male, 88-year-old), and VI (female, 80-year-old); samples from non-demented controls were Braak I (male, 79-year-old), II (female, 94-year-old), and II (female, 88-year-old). The average postmortem interval was 3 h.

Microsomes, coimmunoprecipitation, and liquid-chromatography tandem mass spectrometry. Microsomes were isolated as previously described (Lopez et al., 2007) and modified for the brain (Abisambra et al., 2010). Coimmunoprecipitation (co-IP) was performed as previously described (Jinwal et al., 2012) using anti-Tau46 (Cell Signaling Technology) and anti-actin (Sigma-Aldrich). Protein complexes were identified using liquid-chromatography tandem mass spectrometry (LC-MS/MS) and UNIPROT as previously described (Meier et al., 2015).

In vitro translation assay. 1-Step IVT Kit (Thermo) was used with minor modifications: a black bottom 96-well plate was loaded with *in*

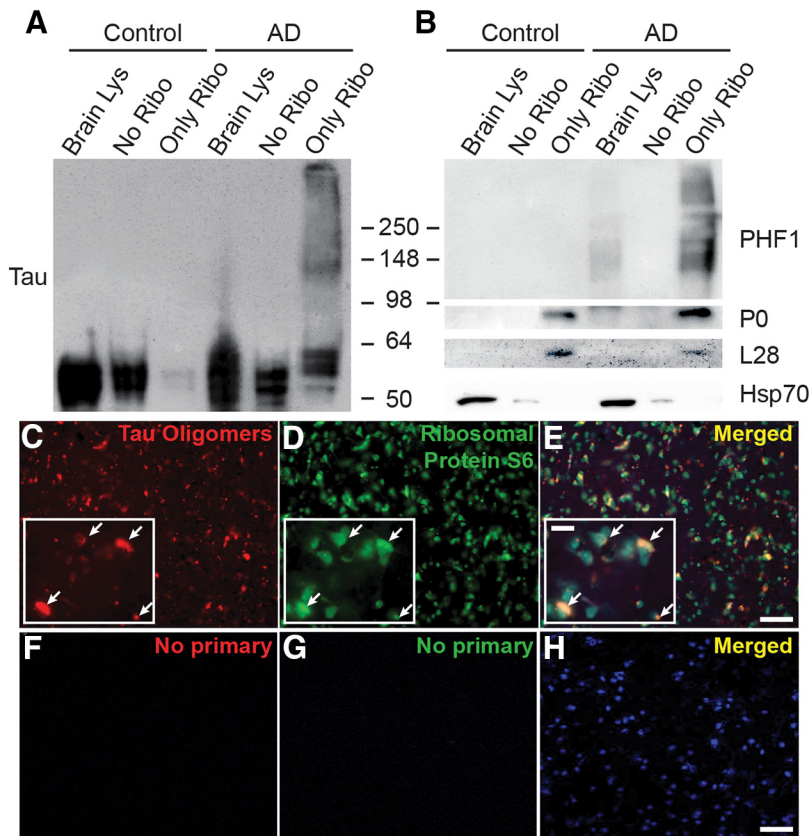


Figure 2. Pathological tau associates closely with ribosomes in AD brains. Representative immunoblots showing total tau (**A**) and PHF1-positive smear (**B**) enriched in AD brain ribosomal fraction. P0 and L28 (ribosomal proteins) and Hsp70 (cytosolic) confirm subcellular fractionation. **C–E**, Representative IF of AD brains showing overlap of oligomeric tau and rpS6 in human AD brain. Co-IF labeling of tau oligomers with T22 (red; **C**), rpS6 (green; **D**), and neurons (Neurotrace; blue). **E**, Merged inset image shows red and green overlap (arrowheads). **F–H**, Representative images of AD brains in which primary antibody incubation was omitted.

in vitro translation (IVT)-kit components and 10 ng of recombinant proteins. GFP (ex-482 nm/em-512 nm) was measured every 15 min in a BioTek Synergy HT at 30°C for 6 h. Each sample was run in triplicate and analyzed using GraphPad Prism (Student's *t* test).

Cell culture, primary neurons, and immunoblotting. Cell maintenance, harvesting, and tau expression were performed as previously described (Abisambra et al., 2012, 2013b). P0–P1 primary neurons were obtained as previously described (Abisambra et al., 2013a). Samples were processed for immunoblotting as described earlier using BCA (Pierce) to estimate protein concentration, tris-glycine gels (Invitrogen), and PVDF membranes (Jones et al., 2011). Primary antibodies: anti-tau h-150 (1:1000; Santa Cruz Biotechnology), actin (1:1000, Sigma-Aldrich), RPL28 and RPP0 (1:1000, GeneTex), PHF1 (1:500), Hsp70 (1:1000, ENZO), PSD-95 (1:1000, Cell Signaling Technology), and puromycin (1:1000, EMD Millipore). Bands were detected using (Pierce ECL). Image analysis was performed using ImageJ. Bands of the protein of interest were normalized to a loading control. Statistical analysis was performed using Student's *t* test in GraphPad Prism.

Surface sensing of translation. Surface sensing of translation (SUNSET) was performed as previously described (Schmidt et al., 2009) with minor modifications: cells were incubated with 10 μ g/ml of puromycin in cell culture media for 1 h before harvest. Proteins were analyzed using immunoblots.

Immunofluorescence. Immunofluorescence (IF) was performed as previously described (Abisambra et al., 2013b). Primary antibodies: T22 (1:100) and RPS6 (1:250; Santa Cruz Biotechnology). Tissues were also stained with Sudan black and Neurotrace (1:200). Slides with AD and control sections were stained omitting primary antibodies to establish nonspecific background signal.

Microscopy. A Nikon Eclipse Ti laser-scanning confocal microscope was used to capture images. Fields analyzed using 40 \times and 100 \times objectives included areas of tau staining with morphologic distribution in agreement with Neurotrace labeling. All acquisition intensities, field sizes, and settings were kept consistent across all images. Images were prepared using the NIS Elements 4.20 (Nikon) and Photoshop Cs6 (Adobe) software and were based upon cells that most closely represented the group.

Quantitative real-time PCR. Total RNA was extracted from rTg4510 tau transgenic and littermate control primary neuronal cultures using EZNA total RNA Kit II according to manufacture instructions (Omega Bio-tek, catalog #R6934-01). RNA was quantified using a BioTek spectrophotometer and cDNA was produced using SuperScript IV (Invitrogen). Quantitative real-time PCR was performed using TaqMan Gene Expression probes for PSD-95 and GAPDH using a ViiA 7 Real Time PCR System (Applied Biosystems). PSD-95 expression was evaluated by normalizing to GAPDH as an internal control. The real-time values for each sample were average and evaluated using the comparative CT method.

Results

We recently determined ER-bound proteins associate with tau (Abisambra et al., 2013b; Meier et al., 2015). To identify tau-associated ER proteins in AD, microsomes were isolated from non-demented control and AD brain tissues. Full-length tau (or actin as control) was co-IP from microsomes, and tau-associated peptides were identified using LC-MS/MS. Of the

216 identified proteins, 68.5% were unique in AD, 22.2% were unique in control, and 9.3% were common between both groups (Fig. 1A). Proteins were grouped into functional categories based on UNIPROT. A striking difference was that tau associated with more RNA-binding proteins in AD than in control (Fig. 1B, C).

To further characterize the tau–ribosome association, we compared tau levels in AD and control subcellular fractions (Fig. 2A). Although tau levels were similar between the fractions lacking ribosomes, tau was significantly increased in the AD ribosomal fraction (Fig. 2A). We also detected a PHF1-positive smear in the AD ribosomes (Fig. 2B). PHF1 recognizes pS396/S404, which is associated with late stage tangles in AD (Greenberg et al., 1992).

Recent studies show that oligomeric tau is highly toxic (Lasagna-Reeves et al., 2011), and it exhibits a prion-like behavior by propagating and seeding (Guo and Lee, 2011). We speculated that if aberrant tau–ribosome complexes were pathogenic, then tau oligomers would associate with ribosomes. To test this, we co-IF labeled tau oligomers and a ribosomal protein, rpS6, in AD and control brain sections (Fig. 2C–H). Confocal imaging revealed that tau oligomers and rpS6 signals overlapped (Fig. 2C–E), suggesting that oligomeric tau associates closely with ribosomes in AD.

The consequences of the tau–ribosome association are unknown. We hypothesized that the aberrant interaction between pathological tau species and ribosomes impairs translation. To test this, we measured the impact of tau on ribosomal function in

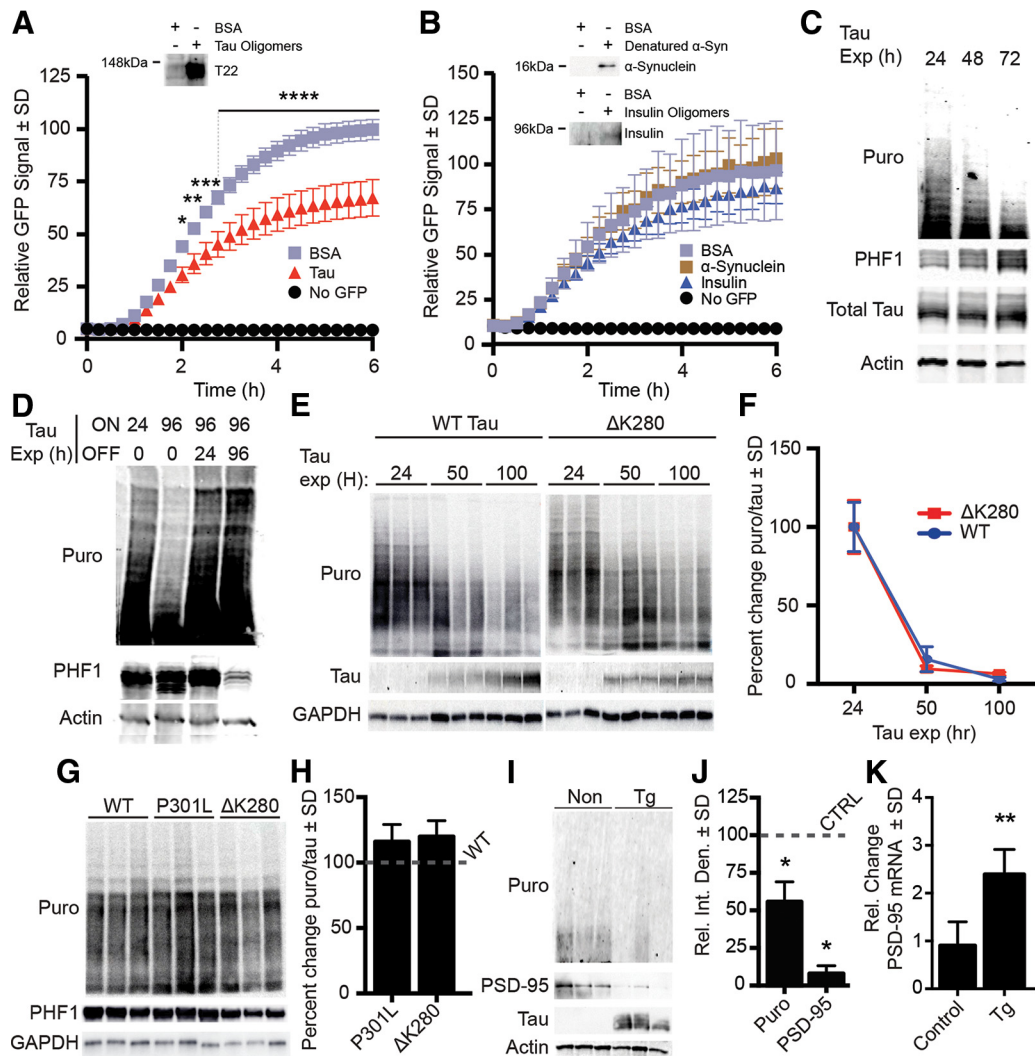


Figure 3. Pathological tau species decrease translation. **A**, IVT graph showing 32% reduction of translational output (after 6 h) in wells with tau oligomers compared with BSA control. **B**, Oligomeric α -synuclein and insulin did not affect translation. Immunoblots confirm the presence of oligomers in the translation assay; α -synuclein samples were denatured to yield monomers because high molecular weight oligomeric conformers are not detectable with anti- α -synuclein antibodies. **C**, Total and PHF1 tau are inversely proportional to the rate of protein synthesis. iHEK-Tau cells were stimulated with tetracycline to express tau for 4 d. Nascent proteins were tagged with puromycin. **D**, Cessation of tau expression rescues protein synthesis. Tau expression was induced with tetracycline for 24 or 96 h (lanes 1 and 2). At 96 h, puromycin levels decreased, whereas PHF1 increased. After continuous tau expression for 96 h (lanes 3 and 4), cells were washed and incubated with media lacking tetracycline, thereby halting tau expression for 24 or 96 h (lanes 3 and 4, respectively). **E**, Representative immunoblots showing the effect of wild-type (WT) and Δ K280 tau on protein synthesis. Changes in tau levels inversely correlated with puromycin. **F**, Quantification of **E** showing no significant difference in levels of protein synthesis between wild-type and Δ K280 mutant tau-expressing cells. **G**, SUNSET comparing the effect of P301L, Δ K280 tau, and WT in transiently transfected HEK293 cells. **H**, Quantification of **G** showing no significant difference between translation levels. **I**, Blot showing that overall translation (Puro) and PSD-95 are decreased in rTg4510 primary neurons. **J**, Quantification of **I**. Puromycin and PSD-95 were significantly decreased in rTg4510 neurons by 43 and 92%, respectively ($*p < 0.05$). **K**, Quantification of PSD-95 mRNA expression from rTg4510 neurons as measured by RT-PCR ($**p = 0.0013$).

three model systems. First, we tested the effect of tau oligomers on translation using a cell-free assay. We added recombinant tau oligomers or bovine serum albumin (BSA) as control with *in vitro* translation assay components and a GFP plasmid reporter. The rate of translation, measured by GFP, was significantly decreased in the presence of tau oligomers (Fig. 3A). To determine whether this effect was specific to tau oligomers, we tested the impact of oligomeric α -synuclein and insulin on translation (Fig. 3B). We found no significant change in GFP, suggesting that this effect is specific to tau.

We next measured the effect of pathological tau on the rate of translation in eukaryotic cells using SUNSET (Schmidt et al., 2009): a puromycin-based pulse assay. Puromycin, which is incorporated into recently translated proteins, was added to medium 1 h before harvesting cells. Nascent proteins, which appear

as a smear, can then be quantified via immunoblots with anti-puromycin antibodies.

We used iHEK-Tau cells, an inducible HEK line that overexpresses wild-type human 4R0N tau upon addition of tetracycline (Abisambra et al., 2013b). Use of tetracycline allowed control over the start and overall duration of tau expression. We first performed a time course experiment in which tetracycline was added to iHEK-Tau cells over 72 h. We found that increased PHF1 and total tau levels correlated with decreased puromycin signal (Fig. 3C). We then performed a rescue experiment in which tau was expressed for 96 h and then tau expression was turned off for 24 or 96 h (Fig. 3D). We found that puromycin levels were rescued back to normal control once PHF1 levels were reduced.

Because mutations on tau are associated with risk for many tauopathies, we sought to determine whether mutant tau variants

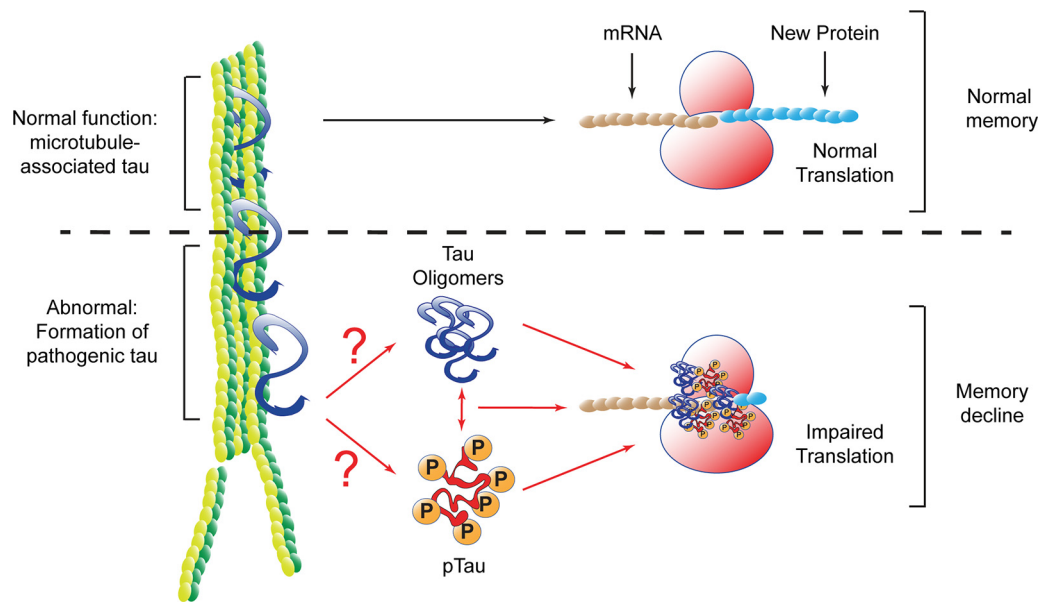


Figure 4. Schematic representation showing the consequences of pathological tau association with ribosomes. Under normal conditions, tau promotes tubulin polymerization and stabilizes microtubules. *De novo* protein synthesis, and in particular nascent neurotransmitter production is necessary for normal learning and memory. Our data suggest that in tauopathic brains, tau adopts aberrant conformations that associate with ribosomes. This interaction reduces nascent protein synthesis. Abrogation of new proteins impairs memory. This mechanism links the most common symptom of tauopathies.

inhibited protein synthesis. To this end, we performed SUnSET in iHEK cells that overexpress the disease-associated Δ K280 tau (Rosso et al., 2003). We found that expression of WT or Δ K280 tau decreased protein synthesis (Fig. 3E), and that this reduction was not significantly different between the two cell lines (Fig. 3F). We then compared translation levels between mutant forms of tau by transiently transfecting HEK293 cells with WT, Δ K280, or P301L tau plasmids. We found no significant difference in protein synthesis levels between WT, Δ K280, or P301L-expressing cells (Fig. 3G,H). These data suggest that tau expression and accumulation of hyperphosphorylated tau impairs protein synthesis.

To determine the impact of pathological tau species in a more neurologically relevant model, we measured changes in the rate of translation in primary neurons derived from rTg4510 tau transgenic and control mice using SUnSET. The rTg4510 model overexpresses human P301L mutant tau (Santacruz et al., 2005). At 14 DIV, puromycin was added to the medium and primary neurons were harvested. Puromycin-tagged proteins were decreased by 43% in rTg4510 neurons ($*p = 0.011$) compared with control (Fig. 3I,J). We then investigated whether a more direct correlate of synaptic integrity could be affected by pathological tau in this model. To this end, we measured the levels of the PSD-95 and found that it was reduced by 92% in rTg4510 neurons ($*p = 0.049$; Fig. 3I,J). We hypothesized that tau-mediated disruption of RNA translation would result in increased mRNA levels. We performed RT-PCR and found that PSD-95 cDNA was increased by twofold in rTg4510 primary neurons compared with controls (Fig. 3K). These data suggest that P301L tau impairs RNA translation, and that this pathological mechanism directly affects translation without impairing gene expression.

Discussion

This study couples cellular and biochemical approaches with proteomics to expand the understanding of the ER-specific tau interactome. The unbiased proteomics approach (Fig. 1) identified 216 tau-associated ER proteins; many of which were unreported. The largest change corresponded to an increase in the

association of ribosomal proteins with tau in AD. Using three *in vitro* models, we show that protein synthesis was significantly decreased as a consequence of the aberrant tau–ribosome association. Although it has been previously reported that tau interacted with ribosomes (Papasozomenos and Binder, 1987; Nelson et al., 1993), the functional consequences of this interaction was unknown until now. We have identified a dysfunctional consequence of tau–ribosome association that impairs protein synthesis, providing the first steps to understanding the mechanism delineating cognitive decline symptoms in tauopathic patients (Fig. 4).

LC-MS/MS results suggest that many other ER proteins (216 total) associate with tau. The use of mass spectrometry for identification of protein–protein interactions is limited and only provides a “first pass” suggestion. For example, whether these interactions are direct, indirect, or false-positives need to be investigated further. As shown in Figure 2, we validated the interaction between tau and ribosomes, and the validation of other interactions is currently underway. When comparing the RNA-binding proteins that associate with tau, we did not find elongation or initiation factors associating with tau in AD samples (Fig. 1C). These data suggest that the association of pathological tau with ribosomes abrogates the recruitment of translation factors to the tau–ribosome complex, and this could lead to reduced translation.

To avoid false-positive results from the LC-MS/MS, we implemented rigorous exclusion criteria (Meier et al., 2015), which omitted well known tau-associated proteins, but increased confidence in these interactions. Our co-IP focused on identifying mature, and not nascent, tau. We used a tau antibody (Tau46) that recognizes the carboxy-terminal tau sequence (404–441). As such this approach obviates caspase-cleaved tau, which is cleaved at D421 and is implicated in the formation of neurofibrillary tangles in AD (Rissman et al., 2004). Future efforts to identify cleaved tau-associated ER proteins might determine novel pathologic mechanisms. Nonetheless, our current list does provide fur-

ther information about the diverse interactions of tau and other ER proteins.

Although tau mutations are not typically associated with risk for AD, there are 48 tau mutations that are associated with onset of other tauopathies (for review, see Zhang et al., 2015). The defining pathologic hallmark of tangles in AD is hyperphosphorylated tau (Grundke-Iqbal et al., 1986). Interestingly, we found that protein synthesis was impaired equally by WT and two disease-associated mutant tau variants: P301L and Δ K280. P301L tau is most commonly associated with frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17; Clark et al., 1998). Expression of this form of tau in the rTg4510 transgenic mice leads to earlier onset and robust neurofibrillary tangle formation (Santacruz et al., 2005). The Δ K280 tau mutant is also associated with FTDP-17 (Rosso et al., 2003), as well as AD (Momeni et al., 2009). The presence of this mutation decreases tau's ability to bind to microtubules (Rizzu et al., 1999), and leads to increased levels of tau aggregation (Barghorn et al., 2000). Our findings indicate that tau-mediated impairment of protein synthesis could be a common mechanism of neuronal dysfunction between tauopathies (Fig. 4).

Our data support the hypothesis that pathological tau specifically reduces ribosomal function, which could lead to memory alterations in tauopathies. We also found that PSD-95, a synaptic protein that participates in learning and memory (Migaud et al., 1998), was decreased in rTg4510 neurons (Fig. 3*I,J*). Cognitive impairment seen in tauopathy models could be attributed not only to reduced protein synthesis, but also to targeted decrease of synaptic proteins.

Interestingly, oligomers of other proteins (α -synuclein and insulin) did not alter ribosomal function (Fig. 3*B*). This finding suggests that there is a mechanism of ribosomal downregulation that specifically implicates tau, but not all other oligomeric and pathologically altered proteins. However, it is possible that proteins involved in the pathogenic mechanisms of other neurodegenerative disorders such as $A\beta$, poly-glutamines, and TDP-43 could also inhibit *de novo* protein synthesis. Experiments to test the effects of these oligomers are currently underway.

Characterization of this ribosome-directed molecular mechanism of tauopathies could provide novel therapeutic opportunities. For instance, therapeutic strategies aiming to uncouple pathological tau from the ribosome might restore RNA translation and prove effective to treat AD and other tauopathies. To do this, specific ribosomal proteins that associate with tau need to be identified as well as the discreet regions where tau binds to these proteins. This would guide the proof-of-concept use of peptides resembling these amino acid stretches to outcompete tau from associating with the ribosome and thereby restore protein synthesis.

This work suggests a direct effect of tau on translation by its association with ribosomes; however, there is also an indirect relationship between tau and translation. One of these is the chronic activation of the protein kinase RNA-like endoplasmic reticulum kinase (PERK). Accumulation of tau impairs ER-associated degradation, which then activates the unfolded protein response and subsequently the PERK pathway (Abisambra et al., 2013b). The prolonged activation of the PERK pathway leads to a reduction in RNA translation through phosphorylation of the initiation factor eIF2 α (Marciniak et al., 2006). This alteration could be a cumulative result of tau's direct and indirect effects on translation. Our study provides further evidence that tau's involvement in disease is multi-faceted, that pathological tau heavily affects translation of vital proteins, and that the tau-

ribosome complex could serve as a key therapeutic target for tauopathies.

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